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QUARTERLY

Chlorination of *N*-acetyltyrosine with HOCl, chloramines, and myeloperoxidase-hydrogen peroxide-chloride system

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***N*-acetyl-L-tyrosine (*N*-acTyr), with the alpha amine residue blocked by acetylation, can mimic the reactivity of exposed tyrosyl residues incorporated into polypeptides. In this study chlorination of *N*-acTyr residue at positions 3 and 5 in reactions with NaOCl, chloramines and the myeloperoxidase (MPO)-H_2O_2-Cl^- chlorinating system were invesigated. The reaction of *N*-acTyr with HOCl/OCl^- depends on the reactant concentration ratio employed. At the OCl^-/*N*-acTyr (molar) ratio 1 : 4 and pH 5.0 the chlorination reaction yield is about 96% and 3-chlorotyrosine is the predominant reaction product. At the OCl^-/*N*-acTyr molar ratio 1 : 1.1 both 3-chlorotyrosine and 3,5-dichlorotyrosine are formed. The yield of tyrosine chlorination depends also on pH, amounting to 100% at pH 5.5, 91% at pH 4.5 and 66% at pH 3.0. Replacing HOCl/OCl^- by leucine/chloramine or alanine/chloramine in the reaction system, at pH 4.5 and 7.4, produces trace amount of 3-chlorotyrosine with the reaction yield of about 2% only. Employing the MPO-H_2O_2-Cl^- chlorinating system at pH 5.4, production of a small amount of *N*-acTyr 3-chloroderivative was observed, but the reaction yield was low due to the rapid inactivation of MPO in the reaction system. The study results indicate that direct chlorination of tyrosyl residues which are not incorporated into the polypeptide structure occurs with excess HOCl/OCl^- in acidic media. Due to the inability of the myeloperoxidase-H_2O_2-Cl^- system to produce high enough HOCl concentrations, the MPO-mediated tyrosyl residue chlorination is not effective. Semistable amino-acid chloramines also appeared not effective as chlorine donors in direct tyrosyl chlorination.**

Hypochlorite treatment of proteins and peptides effects chlorination of some tyrosine residues (Domingan *et al.,* 1995; Kettle, 1996). However, hypochlorite chlorination of free tyrosine or tyrosyl residues incorporated into small peptides is complicated by reactions of HOCl/OCl^- with amino groups and/or other reductants present in the experimental systems. Therefore substitution of chlorine to tyrosine does not occur or occurs rather due to secondary reactions (Domingan *et al.*, 1995). Hypochlorite and chloramines are chlorinating agents in biological systems (Heinecke,

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Abbreviations: MPO, myeloperoxidase; *N*-acTyr, *N*-acetyltyrosine; HPLC, high performance liquid chromatography.

1999). Chlorinated tyrosyl residues can be detected in HOCl treated albumin (Kettle, 1996) and in certain peptides such as Gly-Gly-Tyr-Arg (Domingan *et al.,* 1995). On the other hand, tyrosyl residues do not compete with other possible reductants or chlorine acceptors for hypochlorite (Drożdż *et al.,* 1988), which supports earlier results indicating that direct tyrosine chlorination does not occur (Pereira *et al.,* 1973). Thus, there are suggestions that tyrosine biological chlorination is restricted only to several peptides and proteins in which neighbouring amino-acid residues may activate the tyrosyl residue. The other possibility is that a specific intermediary process promotes active chlorine location in the tyrosyl aromatic moiety (Domingan *et al.,* 1995; Kettle, 1996). This paper presents evidence of direct interactions of *N*-acetyltyrosine with hypochlorite at various HOCl concentrations and pH values.

MATERIALS AND METHODS

The study was carried out using *N*-acetyltyrosine and the following chlorinating systems: hypochlorite, chloramines, and the enzyme system composed of myeloperoxidase (MPO), Cl^- and hydrogen peroxide. The MPO used was obtained from human neutrophilic granulocytes isolated from the blood of patients with chronic myelogenous leukemia as described elsewhere (Drożdż *et al.,* 1988). The enzyme was stored at –25°C. The concentration of MPO was estimated spectrophotometrically by measuring light absorption at 430 nm, assuming the molar absorption coefficient equal to 89 $mM^{-1}cm^{-1}$. MPO activity was assayed by guaiacol oxidation at pH 7.0, assuming 1 unit of MPO activity (1 U) as the amount of enzyme which causes an increase in 470 nm light absorption of 1.0 per minute at 25°C. The MPO preparations used had specific activity equal to 22.5×10^9 U/l and A_{430}/A_{280} ratio 0.79.

N-Acetylated derivatives of 3-chlorotyrosine and 3,5-dichlorotyrosine were obtained by the Norman (1967) method using the procedure described previously (Drożdż *et al.,* 1988). Reaction products were studied comparing HPLC separation patterns with those of standards. Major fraction of amino acids employed was converted into acetylated derivatives, however, a small fraction remained as free amino acids.

Sodium hypochlorite (NaOCl) was obtained by saturating NaOH water solution with gaseous chlorine. The obtained NaOCl concentration was assessed spectrophotometrically, measuring A_{295}, assuming that hypochlorite molar absorption equals to 350 M^{-1} cm^{-1}. The required NaOCl concentrations in working solutions were obtained by about 10-fold dilution of 0.92 M NaOCl with water solution of 30 mM citric acid, 27.7 mM acetic acid and 50 mM sodium chloride brought to pH 4.5 (or other pH if required) with 5 M NaOH (solvent A). Hydrogen peroxide (H_2O_2) concentrations were assessed spectrophotometrically at 295 nm, assuming molar absorption equal to 72.4 $M^{-1}cm^{-1}$.

Chlorination of *N*-acetyl-L-tyrosine was carried out in solvent A, or in a solution obtained by mixing solvent A with methanol at a ratio 8:2 (v/v) (solvent B). This solution was used as solvent for high peformance liquid chromatography (HPLC). Borate buffer was prepared by adding 0.2 M H_3BO_3 to 0.05 M $Na_2B_4O_7$ in proportions necessary to obtain the required pH.

N-Acetyl-L-tyrosine was treated with NaOCl or chloramines of alanine and leucine by adding the chlorinating agent to *N*-acetyl-L-tyrosine solution at the given pH. The effects of various NaOCl concentrations on the chlorination of *N*-acetyl-L-tyrosine at pH, 4.5, were studied using 20 mM *N*-acetyl-L-tyrosine solution in solvent A. The reaction was carried out by adding 20 μl 0.092 M NaOCl to 1 ml 20 mM *N*-acetyl-L-tyrosine solution. The pH of the reacting mixture was immediately adjusted to 4.5 by adding citric acid solution of 40.0 g/l. The samples were left for 24 h at room temperature (22°C) and then the reaction products were analyzed by HPLC applying 50 μl of sample on the column. Chlorination with NaOCl at various pH was carried out as described above, but with pH adjusted to the required value by microtitration with citric acid or with 5 M NaOH.

Chlorination of *N*-acetyl-L-tyrosine by chloramines was studied in the following systems:

200 μl of 90 mM *N*-chloramine of alanine or 200 μl of 90 mM *N*-chloramine of leucine was added to 1 ml of 20 mM *N*-acetyl-L-tyrosine in solvent A (pH, 4.5) and the pH of the mixture was set at 4.5 with 1 M citric acid. Similarly, to 1 ml of 20 mM *N*-acetyl-L-tyrosine in solvent A (pH, 4.5) 200 μl alanine/chloramine or 200 μl leucine/chloramine was added and the pH of the mixture was brought to 7.4 with 1 M phosphoric acid. The reactions were carried out at 22°C for 24 h.

High performance liquid column chromatography was carried out using a Kipp Analytica 9224 HPLC Solvent Programmer, Beckman Model 110A pumps and an LKB-Bromma AB 2140 Rapid Spectral Detector with a measuring range from 190 to 300 nm. Octadecyl=Si 100 4.6 mm × 250 mm columns (Serva-Feinbiochemica, Heidelberg, Germany) and solvent B as a moving phase were used. The elution rate was 1 ml/min. Retention times and absorption spectra were measured for the following standard compounds: L-tyrosine (Merck Darmstadt), *N*-acetyl-L-tyrosine (Sigma), 3-chloro-tyrosine (Koch-Light) and 3,5-dichlorotyrosine (Dróżdż *et al.,* 1988). The *N*-acetyl derivatives of 3-chlorotyrosine and 3,5-dichlorotyrosine were synthesized prior to experiments. Methanol (p.a. grade) used for solvents was from Zakłady Chemiczne Oświęcim (Poland). The reaction products studied were identified by comparing their elution patterns and spectra with those of appropriate standards.

Spectrophotometric measurements were performed using a Gilford Response spectrophotometer. Measurements were performed in duplicate and the results were calculated as mean values. Distilled and deionised water was used in all working solutions.

Leucine chloramine and alanine chloramine were obtained prior to experiments by adding 110 μl of 100 μM L-α alanine or 100 mM L-leucine in solvent A (pH, 2.6) to 1 ml of 0.092 M NaOCl.

RESULTS AND DISCUSSION

HPLC separation of *N*-acetyl-L-tyrosine – HOCl reaction products shows the presence of several compounds; that denominated as peak 4, with the retention time 5.21 min and the maximum of absorption at 273 nm, which corresponded to excess *N*-acetyl-L-tyrosine was present in all experiments. The fraction denominated as peak 6, with the retention time of 9.13 min and the maximum of absorption at 279 nm, corresponds to *N*-acetyl-3-chlorotyrosine. Small peak with the retention time 17.37 min and the maximum absorption at 284 nm corresponds to *N*-acetyl-3,5-dichlorotyrosine (Fig. 1).

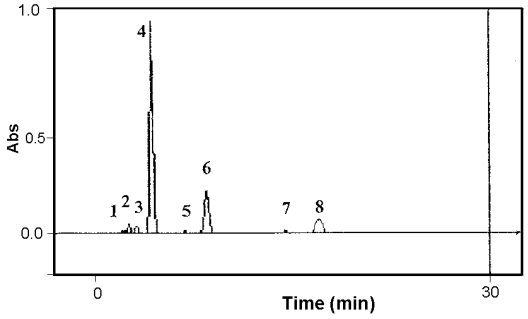

Figure 1. HPLC separation pattern of NaOCl – *N*-acetyltyrosine reaction products.

Peak 6 corresponds to *N*-acetyl-3-chlorotyrosine (retention time 9.13 min). Peak 8 (retention time 17.37 min) corresponds to *N*-acetyl-3,5-dichlorotyrosine. In addition all separations contain peak 4 representing the unreacted *N*-acTyr (retention time 5.21 min).

Production of *N*-acTyr chloroderivatives depends on the HOCl/OCl^- to *N*-acTyr molar ratio. At the HOCl/OCl^-:*N*-acTyr molar ratio 1:10, 3-chlorotyrosine is the only reaction product. At the ratio 1:1 20% of hypochlorite employed is used producing *N*-acetyl-3,5-dichlorotyrosine. Increasing the HOCl/OCl^- to *N*-acTyr ratio causes an increase in 3,5-dichlorotyrosine production, but a decrease of the total chlorination yield. This manifests itself as a decrease in HOCl-chlorine percentage incorporated into the tyrosine aromatic ring. At the HOCl/OCl^- to *N*-acTyr ratio of 1:4, 91% of chlorine is recovered in chlorotyrosine, but at the 1:1 ratio only 35% of chlorine incorporates into tyrosine. No other chloroderivatives were found in the reaction mixture (Table 1).

N-acTyr chlorination depends on the pH of the reaction medium. At the concentration ratio of

HOCl to *N*-acTyr equalling 1:4 and pH 5.5, the highest reaction yield, approaching 100%, was observed. Both lower and higher pH values decrease the yield of 3-chlorotyrosine formation, amounting at pH 3.0 to 66% and at pH 4.5 to 91% (Table 1). Replacement of HOCl by leucine/chloramine and alanine/chloramine at pH 4.5 effects 3-chlorotyrosine production at only 2% of the amount of chloramine chlorine employed. No 3,5-dichlorotyrosine was found (Table 2, Fig. 2).

Table 1. *N*-acTyr chlorination with HOCl/OCl^-

HOCl added (μmol/sample)	pH	3-chloro-*N*-acTyr (μmol)	3,5-dichloro-N-acTyr (μmol)	Chlorination yield (%)
1.8	4.5	1	0	56 ± 4
5.5	3.0	2.74	0.44	66 ± 4
5.5	4.5	3.88	0.56	91 ± 3
5.5	5.5	3.34	1.12	101 ±4
9.2	4.5	4.66	0.70	66 ± 7
12.9	4.5	5.1	1.08	57 ±6
18.4	4.5	3.96	1.22	35 ±9

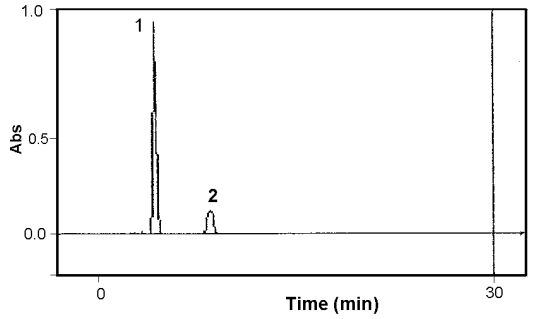

Figure 2. HPLC separation pattern of chloramines — *N*-acetyltyrosine reaction products.

Peak corresponding to *N*-acetyl-3-chlorotyrosine (retention time 9.13 min) is the only reaction product. Peak 1 is the unreacted *N*-acTyr (retention time 5.21 min).

In former studies reactions of deamination and decarboxylation of the unstable tyrosine chloramine complicated the assessement of tyrosine chlorination. The use of *N*-acTyr as the substrate for hypochlorite chlorination in this study makes further studies possible on the chlorination of tyrosyl aromatic moiety without the competing hypochlorite reaction with the tyrosine amino group. Our studies demonstrated chlorination of tyrosine aromatic moiety by HOCl/OCl^-. The reaction, however, requires adequate exposition time and hypochlorite *versus* tyrosine residues concentrations in the range from 1:10 to 1:1. In biological systems the presence of such high hypochlorite concentrations is not likely due to its immediate scavenging by various reductants such as thiols, amines and tryptophanyl residues (Drożdż *et al.,* 1988). MPO-mediated tyrosine chlorination in isolated reaction systems is not effective due to the rapid MPO self inactivation in the absence of active HOCl reductants (Naskalski, 1977).

The mechanism of tyrosine aromatic moiety chlorination is unknown. Tyrosine chlorination has also been achieved using gaseous chlorine water solution in an acidic medium (Thomas *et al,*. 1982). The mechanism employing substitution of elementary chlorine into an aromatic moiety is similar to the well known iodination and bromination reactions:

$$Cl_2 + H\text{-}Ar \rightarrow HCl + Cl\text{-}Ar$$

Thus one may suppose that hypochlorite, which in an acidic medium disproportionates reacting with Cl^-, can produce elementary chlorine in the reaction:

Table 2. *N*-acTyr chlorination with alanine/chloramine and leucine/chloramine

Chloramine added (18 μmol/sample)	pH	3-chloro-N-AcTyr (μmol)	Chlorination yield (%)*
Alanine	4.5	0.24	1.3 ±1.5
Alanine	7.4	0.36	2.0 ±1.5
Leucine	4.5	0.20	1.1±0.8
Leucine	7.4	0.30	1.7 ±2.0

*Mean values from four experiments

$$HOCl + Cl^- + H^+ = Cl_2 + H_2O,$$

which substitutes hydrogen in the aromatic ring, as shown above.

Further studies will be necessary to explain whether this mechanism can be considered in tyrosine chlorination.

Chlorination of tyrosine with NH_2Cl and chloramine taurine was observed by Thomas and coworkers (1986). The results of our experiments showing chlorination of *N*-acTyr with chloramine of L-leucine and chloramine of L-alanine are in accord with the mentioned previous experiments. However, our experiments show that the yield of chloramine mediated chlorination is low, and probably, some additional effects as steric proximity of reagents or additional activation of the tyrosine aromatic moiety are necessary to increase the reaction yield. Chemical modifications of tyrosyl residues in proteins often have a major biological importance in the regulation of activity of intracellular enzymes. The importance of tyrosine chlorination is unknown. Marcinkiewicz *et al.* (1991; 1992) suggest that chlorination of proteins enhances their immunogenicity. Whether tyrosine chlorination is involved in this process is still to be elucidated.

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